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Theriogenology 77 (2012) 873–880
Theriogenology
www.theriojournal.com

Stages and duration of the cycle of the seminiferous epithelium in oncilla (*Leopardus tigrinus*, Schreber, 1775)

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Received 16 October 2010; received in revised form 29 August 2011; accepted 8 September 2011

Abstract

Six adult *Leopardus tigrinus* (oncilla) were studied to characterize stages of the seminiferous epithelium cycle and its relative frequency and duration, as well as morphometric parameters of the testes. Testicular fragments were obtained (incisional biopsy), embedded (glycol methacrylate), and histologic sections examined with light microscopy. The cycle of the seminiferous epithelium was categorized into eight stages (based on the tubular morphology method). The duration of one seminiferous epithelium cycle was 9.19 d, and approximately 41.37 d were required for development of sperm from spermatogonia. On average, diameter of the seminiferous tubules was 228.29 μm, epithelium height was 78.86 μm, and there were 16.99 m of testicular tubules per gram of testis. Body weight averaged 2.589 kg, of which 0.06 and 0.04% were attributed to the testis and seminiferous tubules, respectively. In conclusion, there were eight distinct stages in the seminiferous epithelium, the length of the seminiferous epithelium cycle was close to that in domestic cats and cougars, and testicular and somatic indexes were similar to those of other carnivores of similar size.

Keywords: Spermatogenesis; Feral cats; Seminiferous epithelium cycle; Spermatogenesis; Testis

1. Introduction

Wild cats are among the most endangered species in the world, being affected by many factors, including habitat destruction, food availability, hunting, and low population density [1]. This is a generalized situation for most Neotropical predators, which influences population dynamics and consequently the ecological equilibrium as a whole [2]. The continuous and accelerating loss of global biodiversity has challenged the field of conservation biology, which describes the many factors that influence ecosystems and survival of species [3].

The *Leopardus tigrinus* (oncilla) is the smallest cat species in Brazil; it can weigh up to 3.5 kg, with dimensions and proportions similar to domestic cats [4]. It is an arboreal animal, with a yellow coat and black spots, although some individuals have a melanic pelage variation [4–5]. According to the Brazilian In-

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doi:10.1016/j.theriogenology.2011.09.011

stitute of Environment and Renewable Natural Resources (IBAMA), *L. tigrinus* is classified as vulnerable, and it is also listed in Appendix I of CITES [6].

Spermatogenesis occurs in a spatial and chronologic manner that is highly organized into associations predetermined among various cell types. It may be divided into three phases: 1) proliferative reproductive phase (spermatogonia), when cells undergo rapid and successive mitotic divisions; 2) meiotic phase (spermatocytes), when genetic material is duplicated and genetic recombination occurs; and 3) differentiation or spermiogenic phase (spermatids), when spermatids undergo profound modifications into highly specialized cells (sperm) which are able reach the site of fertilization and fertilize oocytes [7]. The total duration of spermatogenesis, which requires approximately 4.5 cycles, lasts from 30 to 75 d in mammals [8,9], has been generally considered a species-specific constant [10] under the control of the germ cell genotype [11].

The objective of this study was to establish data on reproductive physiology of male oncilla, based on histologic and immunohistochemical analysis of testes, including morphometry of seminiferous tubules, characterization of seminiferous epithelium, and duration of the seminiferous epithelium cycle.

2. Materials and methods

Six adult male *Leopardus tigrinus* from the Center of Wild Animals – UFV (Federal University of Viçosa) situated in the zona da Mata region in the state of Minas Gerais, Brazil, were used in accordance with the IBAMA (authorization Number 15824-1). This study was approved by the Ethics Commission of the Veterinary Department – UFV (Document 85/2007/DVT).

The animals were restrained and anesthetized with a combination of tilethamine chlorydrate and zolazepan chlorydrate (Zoletil; Virbac do Brasil, São Paulo, SP, Brazil) given im (10 mg/kg). The animals were weighed, and the length, width, and thickness of the testis were determined with a digital caliper. Testicular volume was calculated using the formula $4/3\pi$ ABC, where A = half of the width, B = half of the thickness, and C = half of the length of the testis [12–14]. Since measurements were done percutaneously, scrotal skinfold thickness was also measured and subtracted from the testicular dimensions before the calculation. Testicular volume was converted directly into grams, since the volumetric density of mammal testes is very close to unity [15].

Through testicular incisional biopsy using a circular scalpel 2 mm in diameter, a testicular fragment (approximately 3 mm) was obtained and immediately immersed in Karnovsky fixative (4% paraformaldehyde and 4% glutaraldehyde in 0.1-M phosphate buffer, pH 7.4) for 24 h. Thereafter, it was put in 70% alcohol for 24 h before histologic processing. The thickness of the testicular albuginea fragment was measured using a digital caliper (with 10-μm precision). For light microscope studies, fragments were dehydrated in a series of increasing concentrations of ethanol (70, 80, 90, and 100%) and infiltrated with a glycol methacrylate-based plastic resin (historesin, Leica Microsystems, Nussloch, Heidelberg, Germany). As many histologic sections (3 μm thick) as possible were obtained, in sequential manner, using a rotating microtome with glass blade (Leica RM2155, Leica Microsystems, Nussloch, Heidelberg, Germany), and subsequently stained with 1% toluidine blue/sodium borate.

Volumetric proportions of the seminiferous tubules and the intertubular space in the testicular parenchyma were obtained by counting 1,000 points over these tissue components, on digital images from photomicroscopy (Olympus BX 70, Olympus, Tokyo, Japan), in 10 randomly distributed fields for each animal. To calculate the volume of the testicular parenchyma, the volume of the tunica albuginea was subtracted from the total volume calculation. The total volume of each testicular component in all animals was obtained by relating the proportions of seminiferous tubules and intertubular tissue to the total volume of the testicular parenchyma. The mean diameter of the seminiferous tubules and the epithelium thickness were obtained from mean measurements of 10 transverse sections of seminiferous tubules (as circular as possible, in each animal, using the image analysis program *ImageJ* 1.43 (Rasband, 1997–2009, National Institute of Health, Bethesda, MD, USA) [16], using an Olympus BX 70 microscope (Olympus, Tokyo, Japan), at 200 x magnification.

The stages of the seminiferous epithelium cycle (SEC) were characterized using the tubular morphology method [17]. This method was used to evaluate associations among generations of spermatogenic cells at various stages of the seminiferous epithelium cycle. The relative frequency of stages of the SEC were calculated based on the identification and occurrence of each stage in 200 cross sections of seminiferous tubules in each animal.

To calculate the duration of the seminiferous epithelium cycle, intratesticular injections of 0.1 ml of com-

mercial bromodeoxiuridine (BrdU; Zymed Laboratories, Inc., Carlsbad, CA, USA) were given 7 d before the biopsy procedure. A fragment of the testis was dehydrated in a series of increasing concentrations of alcohol (70, 80, 95, and 100%) and subsequently cleared in two consecutive baths of xylene before being embedded in paraffin. The resulting block was sectioned with a rotary microtome, yielding serial sections 5 μm thick. Detection of BrdU was done by staining with a monoclonal antibody. For this, sections were deparaffinized and rehydrated, washed in phosphate buffer (PBS) and peroxidase activity was endogenously blocked with H_2O_2. Then, the slides were washed in PBS, washed in 2n HCl (to denature DNA), and washed again in PBS for enzymatic pretreatment that was performed by incubation in trypsin solution. After washing in PBS, 5% goat serum was used to block cross-reactivity. Then, the material was incubated with biotinylated monoclonal mouse anti-BrdU (Zymed Laboratories, Inc.), which was revealed with a streptavidin peroxidase reaction (Zymed Laboratories, Inc.).

The estimation of the duration of the seminiferous epithelium cycle was performed by observing the most advanced cell in the epithelium; the frequency of the stages gone through from treatment with BrdU to its detection were then calculated. The frequency of the stages completed corresponds to the time spent, and the duration of a cycle of the seminiferous epithelium was calculated.

The combined weight of both testes was used to calculate the gonadosomatic index, the percentage of body mass allocated in the gonads. The tubulesomatic index (somatic allocation in seminiferous tubules), was calculated using the testicular tubular mass and total body weight.

For all data, mean, standard deviation, and coefficient of variation were determined (*Microsoft Office Excel 2003*, Microsoft, Redmond, WA, USA).

3. Results and discussion

The mean body weight of the six animals was 2.589 kg (range, 1.75–3.5 kg), comparable to that described by literature for the same species [4]. Mean testicular volume was 0.76 ml and gonadosomatic index (GSI, i.e., the body mass dedicated to gonads) of the adult oncilla was 0.06% (Table 1). The GSI is directly related to body size, since smaller animals usually have greater allocation and greater energy expenditure in testis mass than larger animals [18]. Among wild carnivores, the largest species, for example, the maned wolf (*Chrysocyon brachyurus*), jaguar (*Parthera onca*), puma (*Puma concolor*) and African lion (*Parthera leo*) have a GSI of 0.04%, 0.034%, 0.03%, and 0.015% [19–21,13], whereas smaller animals, such as the crab-eating fox (*Cerdocyon thous*), the domestic cat (*Felis catus*) and the African wild cat (*Felis silvestres*) have GSI of 0.068%, 0.07% and 0.05%, respectively [18,22,23]. The GSI of oncilla was comparable to those described for other small carnivores.

Table 1
Biometric end points of the testis, volumetric proportion, and gonadosomatic index of captive adult oncilla.

End point	Mean ± SD	Coefficient of variation (%)
Body weight (g)	2589 ± 362	14
Volume of both testis (ml)	1.53 ± 0.386	25.22
Gonadosomatic index (%)	0.06 ± 0.0188	31.32
Testicular albuginea thickness (μm)	250 ± 16.7	6.68
Volume of both albuginea (ml)	0.19 ± 0.047	22.30
Volume of both testicular parenchymas (ml)	1.32 ± 0.35	27.25
Volumetric proportion of the testicular albuginea (%)	13.90 ± 1.87	13.52

The mean thickness of the testicular albuginea of the adult oncilla was approximately 250 μm, and its volume was 0.19 ml, which represents approximately 13.9% of testicular mass (Table 1). In most domestic species, the volumetric proportion of the testicular albuginea and mediastinum is generally ~10% [8]. However, in carnivores, the testicular albuginea appears to be more abundant (18%), as reported in domestic cats, dogs and African lions [14,21,23]. The values obtained for oncilla were very similar to those reported for the crab-eating fox (12.5%) and for the maned wolf (11%) [19,22].

The testicular parenchyma occupied approximately 86.1% of the testis, comprising a volume of 1.32 ml in both testes (Table 1). The testicular parenchyma of the oncilla had approximately 81.29% of its mass in seminiferous tubules and the remainder (8.71%) in intertubular tissue, for approximate volumes of 1.05 and 0.25 ml, respectively. The tubular compartment is the main component of the testis in most mammals, having a great influence on the testicular weight and sperm production [8,24,25]. With the exception of the low values described for the marmot and capybara [7,25], in most animals investigated, seminiferous tubules comprised 70 to 90% of the mass of the testicular parenchyma [13,19–22,26].

Regarding the somatic allocation of seminiferous tubules (tubulesomatic index, TSI) in oncilla, 0.04% of

Table 2
Seminiferous tubules diameter, total length and per gram of testis, seminiferous epithelium height, and tubulesomatic index in oncilla kept in captivity.

End point	Mean ± SD	Coefficient of variation (%)
Seminiferous tubule diameter (μm)	228.29 ± 21.31	9.33
Seminiferous epithelium thickness (μm)	78.86 ± 9.19	11.66
Total length of seminiferous tubules (m)	26.38 ± 9.04	34.26
Seminiferous tubule length per gram of testis (m/g)	16.99 ± 2.56	15.10
Tubulesomatic index (%)	0.04 ± 0.01405	33.94

body weight was allocated in seminiferous tubules (Table 2). The TSI measures the seminiferous tubules in relation to body mass; monogamous or polygenic species have a lesser tubulesomatic investment than promiscuous or polyandric species [22]. The oncilla has a TSI of 0.04%, similar to that described for the crab-eating fox (0.042%) and following the pattern observed for other monogamous or polygenic carnivores [22], which represents a greater investment in sperm production than expected for most of the monogamous or polygenic wild felids. This fact reinforces the need for further studies of reproductive behavior in this species.

On average, diameter of the transverse section of seminiferous tubules was 228.29 μm, whereas height of the seminiferous epithelium was ~78.86 μm (Table 2). The tubular diameter measurement is classical indicator of spermatogenic activity [27–32]. Although mean tubular diameter can reach up to 550 μm in some species of marsupials [33], values for most amniotes range from 180 to 300 μm [34]. The value for oncilla was similar to that of the puma (227.37 μm), maned wolf (227.3 μm), jaguar (257 μm), African lion (252.72 μm), crab-eating fox (236 μm), domestic cat (250 μm), and ocelot (211.35 μm) [13,19,20–23,26].

The average height of seminiferous epithelium mean in the oncilla was 78.86 μm, close to that reported in domestic cat (81 μm) and ocelots (75.4 μm) [23,26], but with a small variation within the group of wild carnivores previously studied: puma 67 μm, jaguar 90.3 μm and African lion 93.2 μm [13,20,21]. However, all values were within the range described for domestic animals (60–100 μm) [8].

The oncilla had 26.38 m of seminiferous tubules in both testes (mean of 16.99 m/g of testis; Table 2). Since testis size varies widely among species, tubular length per gram of testis is more relevant than total length of the testicular. *L. tigtinus* had 16.99 m of testicular tubules per gram of testis, which was lower than that of the domestic cat (23 m/g) [23], greater than jaguar and African lion, 12.2 and 12.4 m/g, respectively [20,21], but similar to many wild carnivores, including the puma (18.2 m/g), maned wolf (18 m/g), crab-eating fox (18.1 m/g), and ocelot (17.81 m/g) [13,19,22,26].

The spermatogenic process in oncilla was categorized in eight stages (Fig. 1), based on the form, presence and location of the nuclei in the spermatogonia, primary spermatocytes and spermatids, as well as meiotic division figures. Overall, these eight stages were similar to those in other mammals.

In this species, the nucleus of the Sertoli had a highly developed nucleolus (present in all stages), approximately 2.5 μm diameter, with flaccid chromatin. Spermatogonia A-Type was present in increasing numbers from Stage 1 through Stage 5, near the basal lamina. In Stage 6, some intermediate spermatogonia were observed; they had a smaller and darker nucleus compared to those of the A-type spermatogonia. In Stage 7, B-type spermatogonia were present, with either a round or ovoid nucleus and greater heterochromatin content. In Stage 8, some preleptotene primary spermatocytes were present replacing B-type spermatogonia. Preleptotene spermatocytes had a smaller nucleus with homogeneous chromatin and one or two nucleoli. In Stage 1, primary spermatocytes (in transition from preleptotene to leptotene stages) were present, and in Stage 2, these cells had typical characteristics of leptotene, with a light cytoplasm and nucleus with clusters of peripheric heterochromatin. In stage, these cells differentiated first into zygotene and then quickly to pachytene. The latter stage of spermatocyte had a larger nucleus with condensed chromosomes, but no evident nucleolus. This cell type was the most common and lasted for a whole cycle, i.e., was present at all stages in sequence to the next stage, three, when it quickly differentiated into a diplotene spermatocyte. The latter was the largest germ cell in the seminiferous epithelium. In Stage 4, there were two meiotic divisions, with metaphyseal plates observed first in diplotene and later in secondary spermatocytes. After the second meiotic division, round spermatids were observed, marking the beginning of Stage 5. Round spermatids were small cells that generally formed three or four layers at the upper part of the seminiferous epithelium, from Stage 5 to the next Stage 1. In Stage 2, spermatids begin the progressive process of elongation, which culminated with a spermatid becoming a spermatozoa that was released in the next Stage 8.

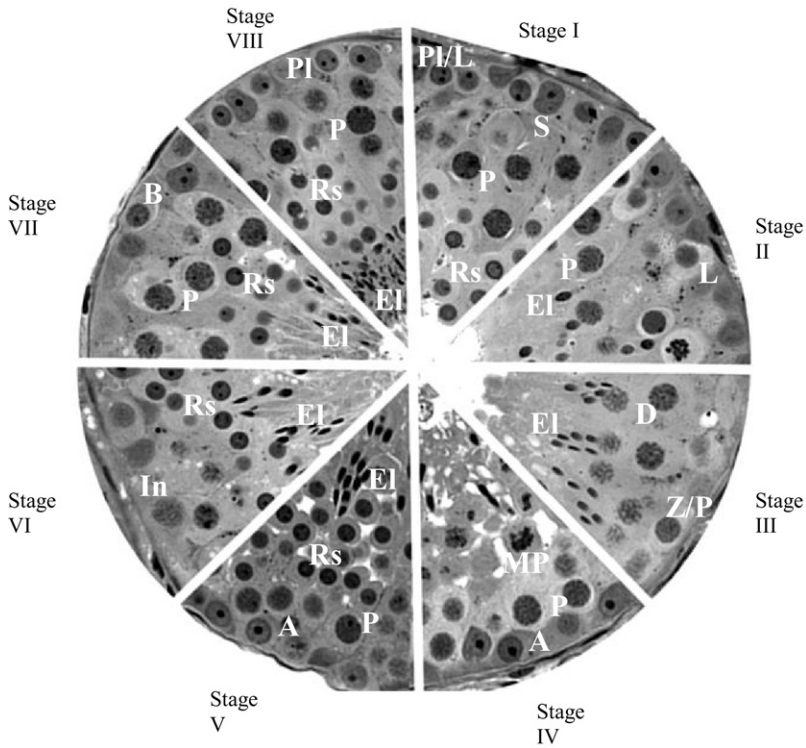

Fig. 1. Photographic mounting showing eight stages of the seminiferous epithelium cycle in oncilla. Sertoli cell (S); A-type spermatogonia (A); intermediate spermatogonia (In); B-type spermatogonia (B); preleptotene spermatocyte (Pl); preleptotene/leptone spermatocyte transition (Pl/L); leptotene spermatocyte (L); zigotene/pachytene spermatocyte transition (Z/P); pachytene spermatocyte (P); diplotene spermatocyte (D); MP; Rs; elongation spermatids (El).

The relative frequency of each stage of the seminiferous epithelium cycle in the oncilla is shown (Fig. 2). Usually stages are grouped into three phases: premeiotic, meiotic, and post-meiotic. Most domestic animals have a higher frequency of the premeiotic phase, in relation to the post-meiotic phase [8]. In oncilla, the premeiotic phase and post meiotic are equivalent (45 and 46%, respectively). In other felids studied (puma and domestic cat) the frequency of the premeiotic phase is also equivalent or slightly longer than the post-meiotic phase [23,35], except for the jaguar which has the longest post-meiotic phase (57.1%) [36]. The duration of the seminiferous epithelium cycle and the relative frequency of these stages are a biological species-specific constant, which are both controlled by the genotype of the germ cells [11], and not affected by any known factor [10,37].

Bromodeoxiuridine is incorporated into the nucleus of the germ cells, which are synthesizing DNA at the moment of the injection, specifically spermatogonia and primary spermatocytes in preleptotene/leptotene in Stage 1 of the seminiferous epithelium cycle. Thus, by collecting the testis fragments at well-defined time intervals after treatment, it is possible to estimate the percentile of the finished cycle as well determine its duration (Fig. 2).

In this study, testis fragments were collected approximately 7 d after treatment with bromodeoxiuridine. The marked cells, which were most advanced in the seminiferous epithelium of these animals, were primary spermatocytes in pachytene at Stage 5 of the seminiferous epithelium cycle (Fig. 3). Within 7 d, there was a progression of ~76.25% (average frequency of the Stages 1–5) in the cycle of the seminiferous epithelium; therefore a cycle was 9.19 ± 0.3 d on average (Fig. 2). Considering that 4.5 seminiferous epithelium cycles are necessary for all spermatogenic processes to be completed, the total length of spermatogenesis in oncilla was estimated as 41.37 d.

In mammals, the shortest duration of the cycle (6.7 d) occurs in the rodent "bank vole" (*Cletheriomys glareolus*) [38], whereas the longest were recorded for opossum (*Didelphis albiventris*) 17.3 d [39] and the Chinese hamster (*Cricetulus griseus*), 17.0 d [40]. In most animals studied, the duration of the spermatogenic cycle ranged from 10 to 14 d for 60%, and 7 to 9 d for

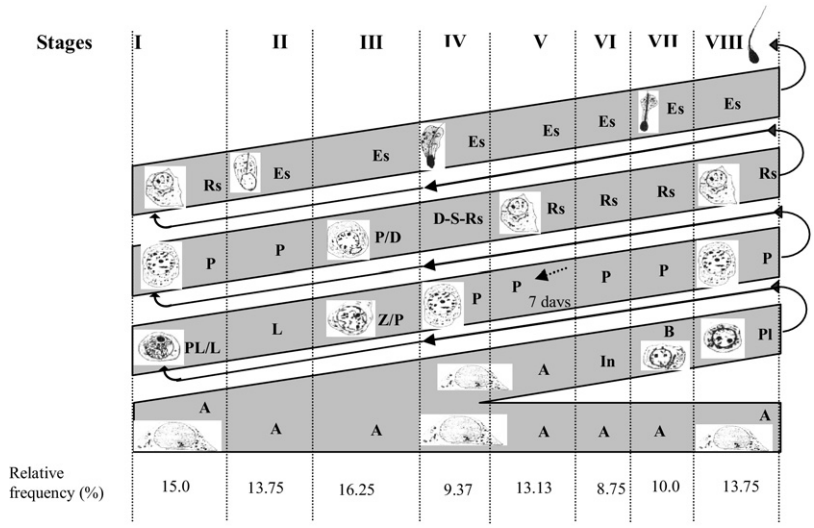

Fig. 2. Diagram of the spermatogenesis process, where each line corresponds to a generation of spermatogenic cells and each column corresponds to one of the eight subsequent stages of the seminiferous epithelium cycle in oncilla. A-type spermatogonia (A); intermediate spermatogonia (In); B-type spermatogonia (B); primary spermatocyte: in Pl; in leptotene (L); in zygotene (Z); in pachytene (P); in diplotene (D); secondary spermatocyte (S); Rs; Es. The marked germinative cell, which were more advanced (arrow) at the eight stages of the cycle, 1 wk after treatment with bromodeoxiuridine, was the primary spermatocyte in pachytene at Stage V.

30%. Thus, the duration of spermatogenesis in the oncilla was within the range for most mammals. In oncilla, the durations of the spermatogenic process, seminiferous epithelium cycle, the spermiogenesis, the meiotic prophase and the other meiosis phases were very close to those observed in the domestic puma and cat [13,23]. In relation to the jaguar, a significant difference was observed, reinforcing the phylogenetic divergence between these species [36].

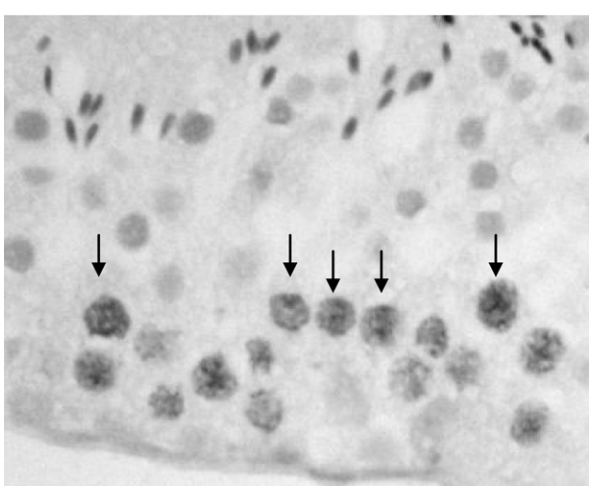

Fig. 3. Pachytene in Stage V of seminiferous epithelium cycle in oncilla; arrows indicate marking with bromodeoxiuridine.

Most of the 36 species of non-domestic felids is threatened or extinct in at least part of their natural occurrence areas [41]. Species living in areas of the tropical forest, where deforestation is intense, are particularly vulnerable. In Brazilian tropical forests, the ocelot (*Leopardus pardalis*), the oncilla (*Leopardus tigrinus*) and the margay (*Leopardus wiedii*) are among the felids struggling to survive in the face of habitat destruction [41,42]. The reproductive physiology of most of the small-sized wild felids has not been studied, especially among Neotropical species. This lack of basic information has made it difficult to develop reproductive techniques and biotechnologies for programs of assisted reproduction in captivity [41].

In conclusion, there were eight stages of the seminiferous epithelium cycle in onchilla, with the length of the seminiferous epithelium cycle (9.19 d) was close to that in most carnivores, especially domestic cats and cougars. The gonadosomatic and tubulesomatic indexes were similar to those reported for other carnivores of similar size. Most of the testis was comprised of seminiferous tubules, with morphometric values similar to other carnivores.

Acknowledgments

We thank the Foundation of Minas Gerais (FAPEMIG) for financial support of this research and the National

Research Council (CNPq) for scholarship productivity grants for graduate students that benefitted the members of our team.

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